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Novel Findings to Aid in Alcohol Use Disorder Research: Ethanol Vapor and the Ventral Tegmental Area

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Novel Findings to Aid in Alcohol Use Disorder Research: Ethanol Vapor and the Ventral Tegmental Area

by

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Dedication

To my grandparents, mother and father, your constant guidance and support was instrumental during this process. I will forever be grateful for your constant advice and encouragement. You all believed in me when I was stressed and unsure. Thank you.

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Novel Findings to Aid in Alcohol Use Disorder Research: Ethanol Vapor and the Ventral Tegmental Area

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The high prevalence of alcohol use disorder continues to impact individuals by heightening stress, anxiety and disrupting cognitive tasks (Morrow and Creese, 1986). Ethanol affects several neuromodulator systems, exerting its effects within the central nervous system (Deehan et al., 2013). One known effect of ethanol is its impact on the mesocorticolimbic system and related circuits, including the ventral tegmental area and its involvement in the regulation of motivation and goal-directed behavior (Doyon et al., 2020). The neurotransmitter norepinephrine has also been linked to mood stabilization, alertness, as well as the endocrine and autonomic nervous systems (Vazey et al., 2018). Additional issues remain to be explored, such as the interaction between norepinephrine signaling and other neuromodulators, and a better mechanistic understanding of ethanol withdrawal (Kushner et al., 2000; Skelly and Weiner 2014; Fredriksson et al., 2015; Becker and Koob, 2016; Petrakis et al., 2016). In order to increase our understanding of alcohol use disorder I carried out two experiments throughout the course of my thesis. We conducted an ethanol vapor study showing that we were able to successfully induce alcohol dependence and withdrawal through a short ten-day vapor exposure model in Long Evans male rats. Lastly, we created a time course of ethanol in the ventral tegmental area through gas chromatography analysis, also using microdialysis. In the appendix we also show pilot data from a study were we closely examined the estrous cycle in

female rats that underwent surgery to place an intracranial guide cannula for microdialysis experiments. We found the possible surgical effects that can halt cycling and ultimately affect data looking at differences between males and females in alcohol dependance and withdrawal, however more experiments are necessary to make a precise conclusion. Findings from these three experiments can be used to continue the advancement of alcohol use disorder research in different ways.

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Introduction

Alcohol Use Disorder

Alcohol Use Disorder (AUD) is defined as a chronic relapsing brain disease characterized by compulsive alcohol use, loss of control over alcohol intake, and a negative emotional state when not using (NIAAA, 2021). Within the United States 15.8 million adults over the age of eighteen suffer from this disease (6.2 percent), resulting in only a startling 6 percent of those with the disorder seeking some sort of treatment in their lifetime (NIAAA, 2021). AUD is a multi-faceted disease that not only affects the individual but also has familial, social and global impacts. In 2010 alone, alcohol misuse costs the United States $249.0 billion dollars and has been globally ranked as the fifth leading risk factor for premature death and disability among adults (NIAAA, 2021).

In the United States specifically, we see a multitude of dangerous drinking behaviors starting from ages as young as twelve, which are than carried out through high school, college and onto adulthood. Binge drinking, the consumption of four drinks or more in two hours in females (five or more drinks in males), is particularly growing in popularity among a variety of age groups (Becker and Koob, 2016). This dangerous behavior is known to increase an individuals' chance of developing an AUD and ultimately contributes to the growing epidemic in the United States.

Individuals with AUD can suffer from a disruption of a number of brain functions including motivation, emotion, and cognition. Alcohol dependence will not only produce an elevated motivation for seeking alcohol but will also negatively impact an individual by heightening stress, anxiety and disrupt cognitive tasks such as decision making (Vazey et al., 2018). The high prevalence of AUD, alongside the wide range of effects of ethanol on biological, physiological, and neurological components has pushed the need for alcohol related

research. Research over the years has aimed to better understand site-specific actions of ethanol on a number of brain regions with the goal to create a treatment that can alleviate the multitude of symptoms that come along with AUD.

Norepinephrine

Norepinephrine is a central nervous system (CNS) neurotransmitter that plays a role in a wide variety of effects on animal and human physiology. The largest populations of noradrenergic neurons are found in the bilateral locus coeruleus (LC) and subcoeruleus found in the dorsal pons. Aside from downward projections to the brainstem and spinal cord the LC provides an array of projections to the cerebral cortex, hippocampus, amygdala, dorsal BNST and some parts of the hypothalamus. Another important cluster of noradrenergic neurons can be found in the nucleus tractus solitarius (NTS), which in turn project to homeostatic and limbic regions of the forebrain (Vazey et al., 2018). This extensive projection pattern throughout the brain sets the groundwork for the global effects that norepinephrine as a regulator of critical homeostatic, emotional and cognitive functions.

In the central nervous system norepinephrine, as stated above, has a vast number of projections throughout the entire brain. As such an important chemical messenger with a global reach norepinephrine plays a profound role in a wide range of behaviors and functions. Norepinephrine has been directly linked to mood stabilization, sleep regulation, aspects of cognition, motivation, and alertness and arousal. In addition, norepinephrine influences the function of the endocrine and autonomic nervous systems (Vazey et al. 2018).

The Role of Norepinephrine in Ethanol Reward

Noradrenergic transmission was found to be a crucial mechanism in the rewarding effects of different classes of drugs of abuse, such as psychostimulants and opioids. In particular it has been suggested that norepinephrine contributes to the attribution of incentive

salience to stimuli associated with drugs (Ventura et al. 2008). Such results could indicate that norepinephrine transmission in the prefrontal cortex is a neural substrate that may also be crucial in the rewarding effects of other drugs such as ethanol.

In the early 2000's, studies began to emerge that reinforced the idea that norepinephrine contributes to ethanol reward. Weinshenker et al. (2000) showed that dopamine beta hydroxylase knockout mice demonstrate a reduced voluntary ethanol consumption, stating that even though this reduction may result from a deficit in ethanol reward, it can also be due to an increased sensitivity to ethanol's aversive effects in the mice. Another study showed that reducing norepinephrine transmission via activation of the $\alpha 2$ autoreceptor decreased alcohol self-administration, whereas increasing norepinephrine via the blockade of this receptor enhanced alcohol self-administration (Le et al., 2005). Lastly, Ventura et al. (2006) has conducted a multitude of experiments examining norepinephrine in relation to the conditioning effects of ethanol, concluding that norepinephrine in the medial prefrontal cortex is necessary to produce ethanol-induced conditioned place preference and for voluntary consumption.

In early human studies, acute ethanol exposure was found to increase norepinephrine and the norepinephrine metabolite 3-methoxy-4-hydroxy-phenylglycol (MHPG) measured in cerebral spinal fluid (CSF) and plasma. The increase in norepinephrine was found to be greater in alcoholic patients than in healthy controls and was connected to blood alcohol levels. The increased levels observed in patients decreased significantly after multiple days of abstinence (Borg et al. 1981; Howes and Reid, 1985).

The Role of Norepinephrine in the Physical Dependence of Ethanol

In alcohol dependent animals receiving daily ethanol gavage, norepinephrine metabolite levels remained high, both while the animals were intoxicated and while they underwent withdrawal, indicating a chronic increase in norepinephrine signaling (Karoum et

al., 1976). Repeated alcohol administration has also been known to sensitize norepinephrine neurons to release larger amounts of norepinephrine (Lanteri et al., 2008). Chronic alcohol exposure was also found to induce c-Fos expression in the nucleus tractus solitarius in males and females (Ryabinin et al., 1997). Additionally, c-Fos signaling was shown to increase in the locus coeruleus of females but not males, potentially indicating some region-specific adaptations in norepinephrine signaling (Retson et al., 2015; Vazey et al., 2018). However, withdrawal from chronic alcohol universally activates norepinephrine signaling across regions (Vilpoux et al., 2009).

Alcohol withdrawal is associated with negative symptoms and is thought to be a main contributor as to why individuals continue to seek out alcohol. There are multiple hypotheses such as that alcohol dependence causes noradrenergic adaptions in the prefrontal cortex that lead to an increase in release of extracellular norepinephrine and an increase in tissue content of norepinephrine during ethanol withdrawal (Vena et al., 2020). Testing these hypotheses about ethanol withdrawal led us to characterize an alcohol dependence model using ethanol vapor. This could lead to examination of a possible time course of withdrawal that could be useful in furthering treatment.

Treatment

Noradrenergic targets have been a big interest among researchers because drugs may be developed to activate or inhibit such targets as a possible treatment for alcohol use disorder. Additional issues remain to be explored, such as the interaction between norepinephrine signaling and other neuromodulators, sex differences in norepinephrine function and better understanding of norepinephrine during withdrawal (Kushner et al., 2000; Skelly and Weiner 2014; Frediksson et al., 2015; Becker and Koob, 2016; Petrakis et al., 2016).

In the periphery, alpha-1 adrenergic receptors are G-protein coupled receptors that are localized postsynaptically in smooth muscle adjacent to nerve terminals and mediate artery and vein vasoconstriction. In the central nervous system activation of this receptor decreases cellular excitability in the temporal cortex and decreases glutamatergic excitatory postsynaptic potential (Haass-Koffler et al. 2018). Several selective alpha-1 adrenergic receptor blockers have already been FDA approved for hypertension and hyperplasia (Eskinder et al. 1989). This has provided an opportunity for researchers to have the ready availability of these medications, such as prazosin and doxazosin, to be tested on animal and human models for alcohol use disorder. The alpha-1 receptor antagonist prazosin is active in the central nervous system and can easily cross the blood-brain barrier, while doxazosin is less penetrable but still holds central nervous system effects (Michel et al. 1989). Prazosin was found to reduce alcohol dependence-induced operant responding during withdrawal but not in the non-dependent rats (Walker et al. 2008). Additionally, studies supported the role of prazosin as a treatment for alcohol use disorder by showing that, in rats selectively bred for alcohol preference, prazosin suppressed alcohol drinking using a two-bottle choice paradigm (Haass-Koffler et al. 2018; Rasmussen et al. 2009). Prazosin in clinical studies has been tested in individuals that have been affected by alcohol use disorder. In a study where prazosin was tested during a six-week pilot randomized clinical trial in individuals seeking treatment for alcohol use disorder (N=24) the prazosin group compared to the placebo group reported fewer drinking days per week and fewer drinks per week. (Haass-Koffler et al. 2018; Simpson et al. 2009).

The alpha-2 adrenergic receptors are G-protein coupled receptors expressed in the central nervous system resulting in sympatholytic effects when stimulated, such as hypnosis, analgesia, and sedation. These responses are thought to be due to the hyperpolarization of noradrenergic neurons and suppression of neural firing (Aghajanian and VanderMaelen, 1982).

Presynaptically the alpha-2 adrenergic receptors also act as autoreceptors acting through a negative feedback mechanism. Clonidine, originally intended as a medication for hypertension, is the oldest FDA approved agonist for the alpha -2 adrenergic receptor. This drug has also been hypothesized to be a potential treatment for alcohol withdrawal for a few decades. Clonidine can cross the blood-brain barrier and acts as an agonist on alpha-2 adrenergic receptors located in the brainstem (Haass-Koffler et al. 2018). Once clonidine passes the blood-brain barrier it is able to decrease norepinephrine release from presynaptic terminals and postsynaptically decreases sympathetic conduction, resulting in lower noradrenergic signaling. In human trials clonidine is known to reduce sedation after a period of heavy alcohol intake, and has also shown to improve the alcohol withdrawal symptoms (Mirijello et al. 2015).

Propranolol, a medication acting on postsynaptic beta-1 and beta-2 adrenergic receptors, has also shown some promising data when treating alcohol use disorder. In a preclinical study where rats were alcohol-dependent through chronic intermittent alcohol vapor inhalation (14 h ON/10 h OFF) and then trained to respond for alcohol in an operant conditioning paradigm on a progressive ratio reinforcement schedule, propranolol was able to decrease alcohol withdrawal induced operant responding for alcohol (Gilpin and Koob, 2010). It has also been found that the combination of prazosin with propranolol reduced alcohol consumption during both alcohol withdrawal and better promoted prolonged abstinence. The combination of these two medications was more effective than single drug treatment in alcohol preferring rats, when given a 2-h free choice between alcohol and water (Rasmussen et al., 2014).

Sex Differences

As of recently sex difference research has gained speed across a wide range of disciplines in the biomedical fields. The idea of sex difference research can be based on a

multitude of concepts; quantitative differences are characterized as the differences in which males and females differ in the magnitude of their response. The trait is the same, but one sex exhibits a greater response than the other. This can be seen in many dose response studies showing that female rats display a greater locomotor response to psychomotor stimulants and more behavioral sensitization than do males (Becker and Koob, 2016). Sex differences in neural mechanisms that mediate behaviors is also possible. Both males and females exhibit a corticotropin-releasing factor-1 (CRF1) receptor response to stimulation with CRF, but there is a difference in the intracellular signaling pathways between males and females (Bangasser and Valentino, 2014). Furthermore, there may also be effects of the estrous cycle and influences of gonadal hormones on neural mechanisms that mediate behavior (Becker and Koob, 2016).

Traditionally data has shown that men drink larger amounts of alcohol compared to women, but recent data has been indicating that the consumption rate for men and women is becoming more similar and has been for the last several decades (Keyes et al. 2008). Differences are especially being seen in the drinking patterns between women and men, more specifically men have more drinking days per month than women. In addition binging episodes have increased overall for females and decreased for males. Both social factors and biological mechanisms have been argued to contribute to sex differences in alcohol drinking patterns, suggesting that women who become addicted progress through initial use to dependence at a faster rate than men (Becker and Koob, 2016). In studies examining alcohol consumption in rats using the two-bottle choice paradigm to examine preference for alcohol over a 24-hour period, female rats of various strains showed greater alcohol intake and preference in two-bottle choice testing (Li and Lumeng, 1984). When measuring the pattern of responding,

studies also showed that females did not decrease intake as much as males when the alcohol concentration was increased (Meliska et al., 1995).

Estrous Cycling

Preclinical studies indicate that gonadal hormones are important factors that regulate drug self-administration in female animals as well as human females. Studies involving human participants have reported increased subjective ratings of euphoria and craving during the follicular phase (high estrogen) compared to the luteal phase (moderate estrogen) following administration of amphetamine and smoked cocaine (Evans and Foltin, 2006). Similarly, studies conducted in rats reveal that drug sensitivity and drug self-administration are dependent on gonadal sex hormones and vary across the rat estrous cycle (Lacy et al., 2016; Caine et al., 2004). Stress, including the stress due to invasive surgical procedures, has been known to affect estrous cycling and could potentially add unwanted confounding variables if proper steps are not taken (Silva et al., 2020). Continuing the involvement of females in future experiments is important for the advancement of the addiction field, however we must be aware of different components that can affect the estrous cycle and ultimately the interpretation of our data. See the appendix for pilot data on how invasive surgical procedure may influence estrous cycling.

Pharmacokinetics

To understand the mechanisms that underlie ethanol-related behavior requires a combination of behavioral analysis along with in vivo measurements of cellular or chemical activity in the brain. Microdialysis has been used in the neuroscience and neuropharmacology fields to monitor neurochemical activity in conscious, freely moving animals. Microdialysis is a technique, in which a semipermeable membrane is implanted into a specific brain region to allow sampling of the local extracellular environment.

There are several ways that microdialysis is useful in alcohol research. For example, microdialysis has been used to examine whether acute ethanol stimulates both the locus coeruleus and the nucleus tractus solitarius noradrenergic neurons (Jaime et al., 2020). Additionally, limited ethanol self-administration experience alters dialysate norepinephrine in the medial prefrontal cortex in a manner consistent with a decrease in tonic norepinephrine release (Jaime et al., 2020). Other experiments used acute intravenous administration of ethanol (1 g/kg) and microdialysis to show an increase of 77% norepinephrine above baseline in the medial prefrontal cortex when compared to controls. Together these studies suggest that ethanol's effects on norepinephrine in brain are related to a stress response rather than the rewarding properties of ethanol (Jaime et al., 2017).

A major theoretical issue while using microdialysis as a technique has been how to calibrate probes to allow prediction of brain concentrations of an analyte from dialysate concentrations. Throughout the field however, it is widely understood that using in vitro calibrations for in vivo measurements is not valid (Gonzales et al., 2002). Although there are difficulties, several quantitative methods are used to gain some insight into mechanisms of actions of ethanol in the brain. One method that has been used is to conduct separate experiments in another group of rats to determine an in vivo extraction fraction for ethanol (Robinson et al., 2000). The in vivo extraction fraction for ethanol can be calculated by computing the ratio of the dialysate ethanol concentration to the blood ethanol concentration. Using this method, the time course for the tissue change in ethanol concentration can be estimated. This method allows us to estimate tissue concentrations after various ethanol doses with the use of a minimal number of animals, unlike ethanol analysis from tissue extractions (Howard et al., 2008).

Chapter 1: Ten Day Ethanol Vapor Exposure Model

Dependence is defined by the physical adaptations that occur with repeated exposures to drugs, while "physical dependence" is most often associated with the presence of withdrawal symptoms and the development of tolerance (Funk et al., 2006). Ethanol vapor exposure has been a preferred method of inducing dependence in rodents in several studies (Funk et al., 2006). It is a non-invasive procedure that allows precise control over the dose, duration, and pattern of exposure. Several studies have used chronic intermittent ethanol (CIE) exposure, in which the animals undergo cycles of alcohol exposure and alcohol abstinence lasting from weeks to months. This procedure has been used to increase self-administration drinking in pre-exposed rats after only 2 weeks of alcohol vapor exposure (O'Dell et al., 2004; Gilpin et al., 2009). In studies where the animals have had no pre-exposure to alcohol, ethanol vapor exposure was used to produce increases in the initiation and maintenance of alcohol self-administration following seven weeks of ethanol exposure (Rimondini et al., 2003; Gilpin et al., 2009).

While the ethanol vapor exposure model has been well known in the field for some time, it has widely been used as a long-term procedure ranging anywhere between four to eight weeks in naïve rodents. With the extended time, previous investigators typically started with low ethanol exposure rates, and the rates were steadily increased to maintain a blood alcohol concentration (BAC) of 0.15 g/dl (Gilpin et al., 2009; Funk et al., 2007). In these longer exposure periods, withdrawal was also documented and was observed for at least 48 hours after exposure (Gilpin et al., 2009). However, it is not known if this extended exposure is necessary to induce alcohol dependence. There is no current data to show the time course for alcohol

dependence development in naïve rats, except in adolescent rats (Healey et al., 2020). Our studies use the same BAC, as well as withdrawal and intoxication criteria used in long term ethanol exposure studies, but instead we aim to examine if alcohol dependence can be successfully induced in a shorter ten-day exposure model in adult male rodents.

Methodology

Species

Long Evans male rats were used in all vapor experiments. Rats were ordered at a weight range of 300.0 -325.0 g (Envigo, Alice, TX). Once the rats were received, each rat was handled for at least ten minutes daily for a week by everyone involved in the experiment. Handling consisted of mimicking movements each rat would experience during intoxication and withdrawal scoring.

V*apor Chambers*

Acrylic chambers measuring 2'x2'x2' (¼ in. thickness) were designed by the Becker Lab (Becker and Lopez, 2004) and manufactured by a local company (Regal Plastics, Austin, TX). Two standard rat cages (17 in. X 8½ in. X 8 in.) were placed in each chamber, in which rodents were single-housed during exposure. Each chamber was connected to two pumps (ethanol: Matala, HK-40L; air: Matala, HK-25L); one to pump fresh air continuously into each chamber, while a second was used to pump alcohol vapor. Ethanol exposure consisted of a 14 hours on/10 hours off model for ten consecutive days (Gilpin et al., 2009). Fresh air and ethanol vapor are pumped at variable rates at a combined 10 L/min, with air continuously being pumped during the ethanol off period.

The ethanol pump was connected by tubing (USP Item #: 54033, 1/4" ID x 3/8" OD Silicone® Tubing) to a y-connector and two on/off valves. One valve was connected to an ethanol bottle (Pharmaco, Ethyl Alcohol 200 Proof, Absolute, Anhydrous ACS/USP Grade) containing three carbonating stones (carbonating stone with 1/4" barb, 2-micron pore size) that generated ethanol vapor when turned on. Vapor from the ethanol bottle was then routed by a one-way valve to a flowmeter (Cole Parmer, PMR1-016598). The flowmeter allowed control of the rates of ethanol that were delivered to the animals. From the flowmeter, the ethanol vapor then flowed through a one-way valve to a y-connector and into the designated ethanol chamber (Illustration 1).

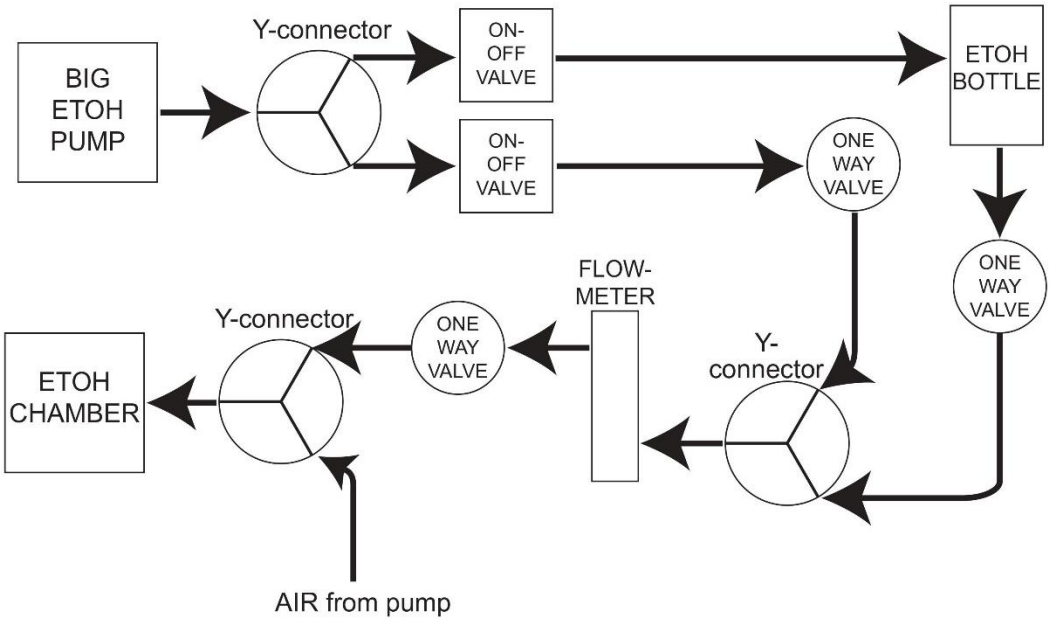

Illustration 1: Ethanol pump to chamber plumbing. See text for details.

The air pump is connected by tubing (USP Item #: 54033, 1/4" ID x 3/8" OD Silicone® Tubing) directly to a flowmeter (Cole Parmer, FR4A41BVBN-CP). This maintained a combined exposure of 10 L/min while the ethanol flowrates varied. From the flowmeter, the air then flowed through more tubing to a y-connector connected to the designated chamber.

Vapor Standards

Vapor standards were made by injecting known quantities of ethanol (Pharmaco, 95% Ethyl Alcohol, Absolute, Anhydrous ACS/USP Grade) into calibrated 1 liter glass bottles with silicone/PTFE septa caps. The aliquots (25 uL, 50 uL, and 75 uL) were injected into three separate glass bottles using a gastight microsyringe (Hamilton, 1725TLL). Ethanol vaporized inside the bottle for thirty minutes at room temperature. A 25 ul microsyringe (syringe: Hamilton, 1725TLL 250ul SYR; needle: Hamilton, RN NDL (22s/2 “/2) S LOT# 647808) was used to sample 15 uL of air from the glass bottles through the septa, and then slowly injected into a 2 ml GC sample vial (septum cap: Agilent, Part# 5182-0722) through the septum. The ethanol in the vapor chambers was sampled in a similar way through the chamber port.

A Bruker 456-GC gas chromatograph with a flame ionization detector and a Varian 8400 autosampler were used to quantify ethanol in vapor and vapor standards, as well as blood and blood standards. Gas chromatography analysis methods can be found in Jaime et al. (2020).

Blood Sampling

Animals were removed from the vapor chambers individually and placed under anesthesia (isoflurane). One back leg of each rat was then shaved to expose the saphenous vein. The saphenous vein was pricked with a lance (Goldenrod Animal Lancet, sterile, 5 mm) from

which 10 uL of blood were collected with a pipette into a 2 ml GC vial prefilled with 90 uL of saturated saline. All blood samples were taken in triplicates. External standards were made using 10 uL of known ethanol concentrations (40 mM, 20 mM, 10 mM, 5 mM) pipetted into GC vials with 90 uL of saturated saline.

Intoxication Scoring

Once the chambers were turned off, each rat was evaluated for severity of intoxication for that exposure day. Long Evans rats were placed on a flat surface that gave them plenty of room to move around and were observed for no longer than a minute. Long Evans rats were then evaluated and scored using an intoxication scale ranging from 1 (normal) to 6 (anesthetic dose) (see Table 1). If a Long Evans rat scored an intoxication score of 5 (loss of righting reflex greater than 30 seconds) a DuraSorb non-sterile underpad (Fisher, NC9325160) was placed over the bedding and a 1 ml injection of saline was given to promote hydration. The rat was monitored, and the surgery pad removed once the rat gained mobility.

Score	**Behavioral Description**
1	Normal
2	Stumbling, no swimming or loss of righting reflex
3	Swimming
4	Loss of righting reflex (greater than 2 seconds, less than 30 seconds)
5	Loss of righting reflex (greater than 30 seconds)
6	No eye blink reflex, anesthetic dose

Table 1. Intoxication Scale for Vapor Exposure

Withdrawal Scoring

On the last day of ethanol vapor exposure, each rat was observed for behavioral signs of ethanol withdrawal at four and eight hours after the chamber was turned off. During observation, each Long Evans rat was placed into a clean home cage and videotaped while live scoring was conducted. Our alcohol withdrawal assessment consisted of observing the following behaviors: posture/gait, tail stiffness, ventromedial distal limb flexion and irritability. Each behavior was scored on a scale from 0 to 2, 0 being normal behavior and 2 being severe (see Table 2). The following scale is modified from Majchrowicz (1975).

Behavior	Withdrawal Scoring		
	0	**1**	**2**
Posture/ Gait	*Normal posture/gait*	*Slight stiffness in hind legs, hunched posture, piloerection*	*Pronounce stiffness and hunched posture such that rat has difficulty walking and is often close to immobile*
Tail Stiffness	*Normal, flaccid tail dragging along bedding*	*Slight but consistent elevation of tail above bedding as animal walks, slight curling of tip of tail upward*	*Tail appears very stiff, substantial upward curling*
Ventromedial Distal Limb Flexion	*No flexion*	*One limb flexed or partial flexion in either or both of limbs*	*Complete flexion of both limbs*
Irritability	*Docile to all handling*	*Docile to touch, when grasped wiggles and makes vocalization*	*Reacts to touch and grasping with vocalization, biting, or rolling over*

Table 2. Withdrawal Behavioral Assessment for Vapor Exposure

Results

Vapor Induction

The flow rate for day one during each vapor exposure cohort was set at a starting ethanol vapor flow rate of 3.2 L/min. This flow was relatively low because the first day was

used to habituate the animals to the vapor chambers. On subsequent days the ethanol flow was increased and adjusted each day based on the intoxication score and BAL. During these nine days the chamber vapor concentration averaged at about 35.3 mg/L, consistent with previous vapor samples analyzed by gas chromatography. It is common for the flow rates to increase and decrease throughout the duration of the experiment- for example, day two averaged at a vapor concentration of 43.26 mg/L while day three averaged at 38.18 mg/L. Technical difficulties with the GC were experienced during both experimental cohorts, which resulted in collecting vapor samples only up to day eight for the first cohort, and day six for the second.

Intoxication scoring was conducted each day after the chambers were turned off (see methodology for intoxication scoring table). Figure 1 shows the average (n=10) intoxication score over the course of the ten-day vapor exposure model. Intoxication was modest throughout exposure in which most of the rats showed signs of staggering. However, a few of our Long Evans rats did experience severe intoxication resulting in a loss of righting reflex. A repeated measures one-way ANOVA was conducted and showed that there was a difference in intoxication scores throughout the ten days of vapor exposure ($F(9,81) = 2.518$, $p < 0.05$). Post hoc analysis showed that the severity of intoxication on day four was significantly different than the first three days of exposure ($F(3,81) = 4.401$, $p < 0.05$). While these analyses showed day four as having a higher intoxication score, our main finding showed that we were able to

induce measurable intoxication throughout the exposure period, even though it is well known that tolerance is being developed.

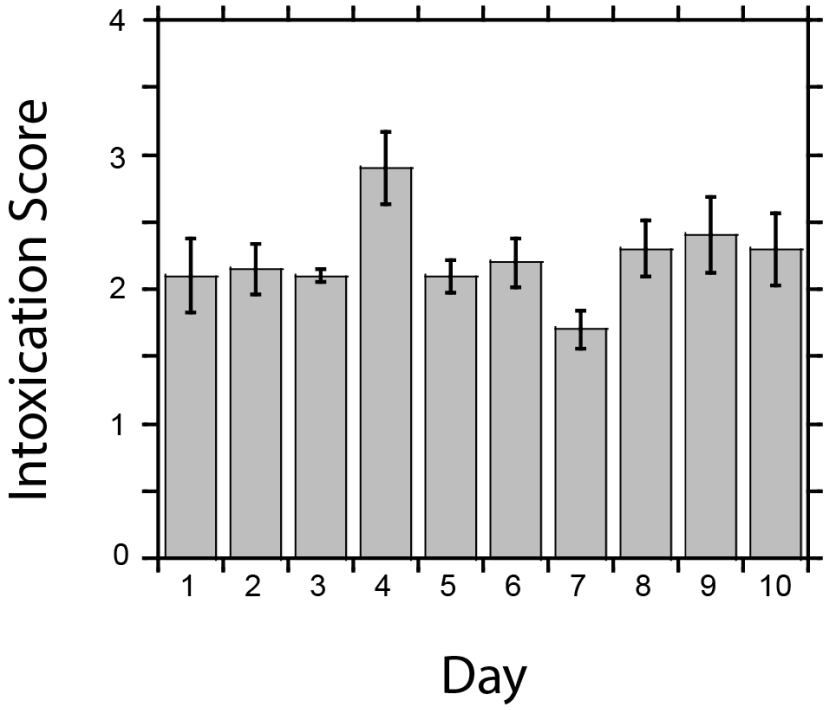

Figure 1. Daily Intoxication Scores. The following graph shows the average intoxication score over the course of a ten-day vapor exposure model. Long Evans rats exposed to ethanol (n =10) are only included (error bars represent the standard error of the mean).

Aside from daily intoxication scoring to assess vapor intoxication, we also took blood alcohol concentration samples three times throughout the ten-day vapor exposure to be analyzed by the GC. The target BAC we used to induce dependence was 0.15 g/dl. Figure 2 shows the average BAC from the three collections during the ten-day vapor exposure (n=8). The graph shows that the target range of 0.15 g/dl was surpassed on each collection day.

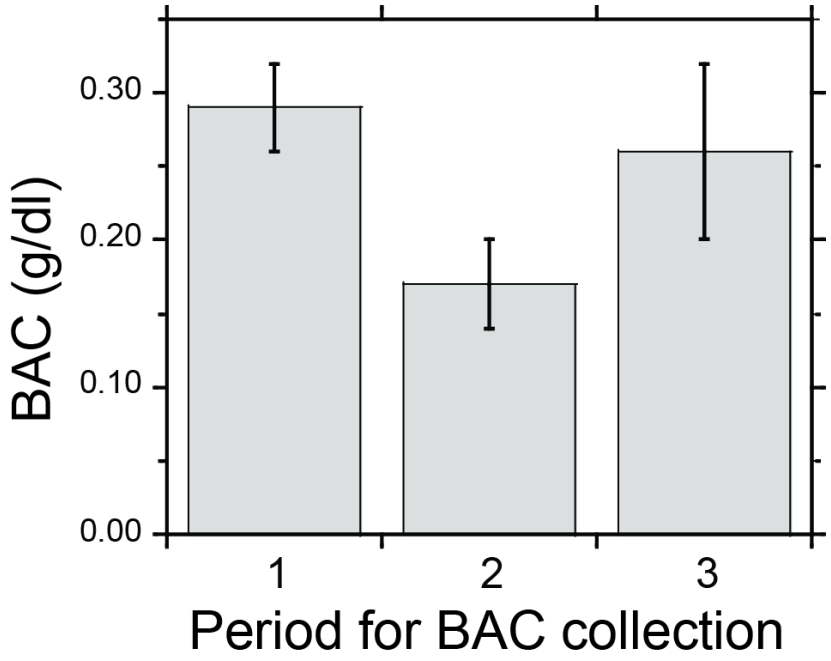

Figure 2: Blood Alcohol Concentrations. BACs are collected three times throughout exposure to maintain a minimum BAC of 0.15 g/dl throughout the ten days of ethanol vapor exposure (n =8). We have been successful in maintaining a minimum BAC to induce alcohol dependence in most Long Evans rats (error bars represent the standard error of the mean).

Withdrawal Assessment

To verify that we successfully induced alcohol dependence with this ten-day vapor exposure model, we assessed withdrawal symptoms in each ethanol and air exposed Long Evans rat. Figure 3 shows the weights of both ethanol (n=8) and air (n=7) exposed animals at pre-exposure and on day 9 of exposure. Notably, while air-exposed animals continued to gain weight throughout the duration of the ten-day exposure, ethanol-exposed rats lost weight. The difference between the ethanol and air exposed rats was substantiated by a one-way ANOVA

in which a significant interaction was detected ($F(1,13) = 8.23$. $p < 0.05$). Further investigation was needed to explore the interaction of exposure day and vapor. A post hoc one-way ANOVA was conducted on day nine weights, which showed that there is a significant effect of exposure on weight ($F(1,13) = 11.78$. $p < 0.05$).

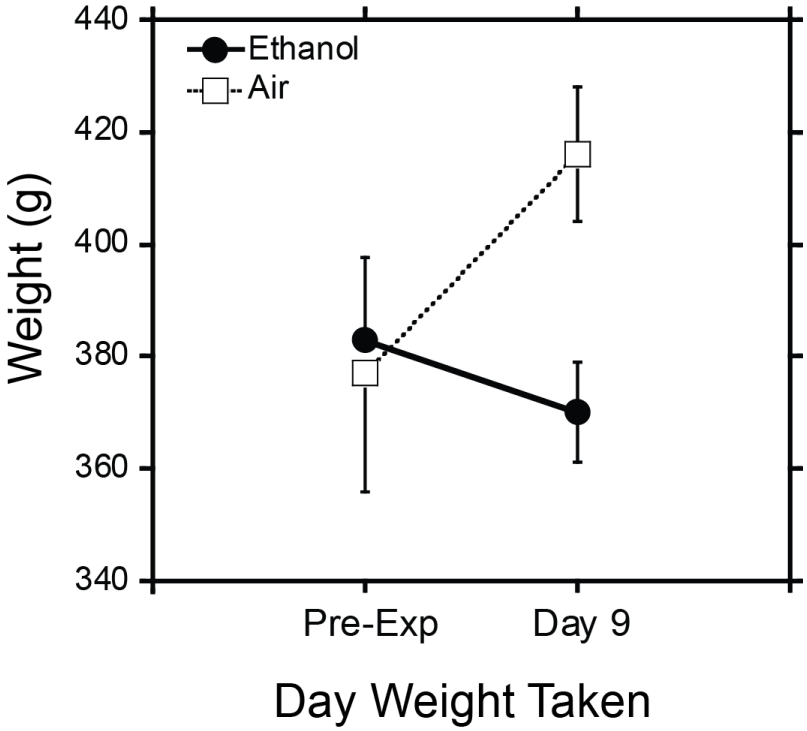

Figure 3: Weight Throughout Ethanol Exposure. Weight in grams in both ethanol and air group pre-exposure and towards the end of exposure (n= 8-9). The ethanol group lost weight throughout exposure, while the air group gained weight (error bars represent the standard error of the mean).

On day ten of our exposure model, both vapor and air exposed Long Evans rats underwent a withdrawal assessment at four and eight hours after the chambers were turned off

(see methodology for withdrawal assessment table). Animals were scored on four behaviors to assess the severity of withdrawal (irritability, ventromedial distal limb flexion, posture/gait, and tail stiffness). Figure 4 shows that ethanol exposed animals (n=8) showed signs of withdrawal at both four and eight hours after exposure. Air exposed animals (n=7) were also assessed but did not show signs of withdrawal at any time point.

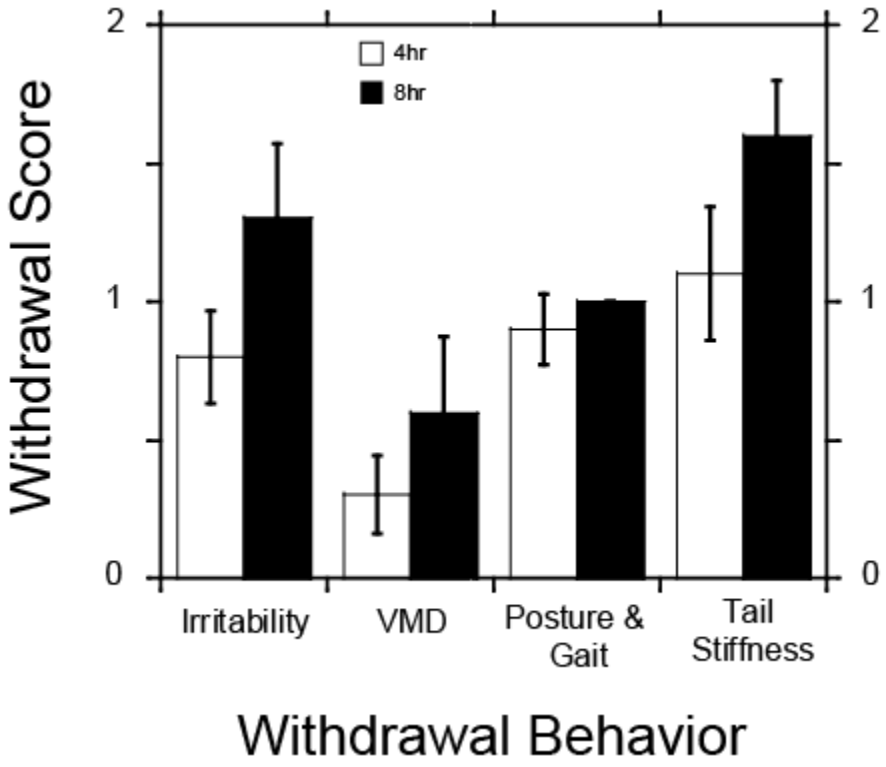

Figure 4: Ethanol Withdrawal Behavior. On day ten of exposure all Long Evans rats underwent a withdrawal assessment to determine if ten days of vapor exposure was successful in inducing alcohol dependence. The following graph shows that ethanol exposed rats (n=8) showed behavioral signs of withdrawal at 4hrs and 8hrs after the last exposure. (error bars represent the standard error of the mean). Air exposed animals are not included since they exhibited normal behavior.

Discussion

The data collected from our ethanol vapor study showed that we were able to successfully induce alcohol dependence and withdrawal through a short ten-day vapor exposure. We were able to maintain all ethanol exposed Long Evans rats throughout the ten days at target BACs of at least 0.15 g/dl. Additionally, ethanol exposed rats showed signs of moderate withdrawal at four and eight hours after the chambers were turned off, with no signs of seizures. It is also important to consider individual differences in each animal that could potentially affect how severely intoxicated they become during each exposure. For example, severe intoxication could ultimately lead to weight loss during the experiment. One possibility to determine the effects of weight loss could be adding a pair feeding group to future experiments.

Previous models for inducing physical alcohol dependence have reported withdrawal behaviors in rodents up to twenty-four hours after there last exposure. For example, Majchrowicz (1975) was one of the first to report observing withdrawal behaviors up to sixteen hours after a high dose gavage model. On the other hand, Gilpin (2009) used a four-week vapor exposure model and stated that all physical signs of withdrawal in rodents subsided after twenty-four hours from the chambers being turned off. As stated above, our ten-day ethanol vapor exposure model was successful in inducing moderate withdrawal at four and eight hours after chambers were turned off. By using a shorter ethanol vapor exposure model, we expected our ethanol exposed animals to be on the lower end of expressing withdrawal behavior. With this in mind, we did not continue to observe the ethanol exposed rats for withdrawal behavior after eight hours from the chambers being turned off.

Using a short ethanol vapor exposure model could be useful in determining cellular and molecular mechanisms in early stages of dependence. For example, it is not known if norepinephrine release is affected during withdrawal. Alcohol withdrawal is associated with negative symptoms and is thought to be a main contributor to the continuation of alcohol-seeking by individuals. Alcohol dependence could potentially cause noradrenergic adaptations that affect the release of extracellular norepinephrine. Future experiments utilizing a short vapor exposure model and microdialysis could potentially explore these possible adaptations caused by ethanol. Funk et al. (2007) used a longer vapor exposure model of four weeks to show that ethanol-dependent animals display enhanced anxiety-like behaviors and enhanced ethanol self-administration during withdrawal, resulting from a dysregulation of corticotropin-releasing factor (CRF) stress systems. Future studies could use a shorter vapor exposure model to determine if adaptations in the CRF stress systems are affected during shorter periods of exposure. Exploring the mechanisms of early CRF and norepinephrine adaptations could lead to a better understanding of the beginning stages of addiction.

Through the duration of the vapor experiment we noticed that ethanol vapor exposed animals lost weight when compared to air exposed Long Evans rats. Gilpin et al. (2009) used a BAC target range of 0.15 g/dl to 0.225 g/dl to limit severe exposure in animals. The authors further mentioned that animals that are unresponsive at the end of exposure often lose a significant amount of body weight. In addition, they suggested general observational guidelines to help prevent severe intoxication should be considered on days that BACs are not collected as follows:

- .20 to .30 g/dl BAC: rat has trouble staying on its feet or cannot stand at all

- .30 to .40 g/dl (or higher) BAC: rat is totally unresponsive

If the intoxication signs reach these levels vapor concentration levels should be adjusted accordingly. Analysis of the weight data in our study also leads to the conclusion that care should be taken to avoid potential overexposure. This can be done by taking weights before vapor exposure begins and following the weight of the animal on a daily basis during the experiment. Weight loss throughout exposure should be considered a sign of consistent severe intoxication.

In the published literature, Trantham-Davidson et al. (2017) used a two day vapor exposure model that was repeated for four cycles in adolescent rats. In this study the adolescent rats were exposed to vapor for a total of eight days, however each exposure period was followed by a period of abstinence. Furthermore, the investigator documented significant intoxication during each exposure day, however they did not report any evidence of withdrawal signs after the fourth cycle. In addition, Gass et al. 2017 used a two week ethanol vapor exposure model in adult rats, but they also did not report whether signs of physical dependance had developed after the vapor was turned off. Our vapor study, however, is currently the shortest vapor exposure model that has been able to successfully induce alcohol dependence over ten days in adult rats. Throughout our study, all ethanol exposed Long Evans rats-maintained intoxication and showed signs of withdrawal at four and eight hours after the chambers were turned off. Our short-term exposure model could be used to investigate the early neurological adaptations caused by ethanol exposure that lead to addiction.

Chapter 2: Time course of Ethanol in the Ventral Tegmental Area

Alcohol use disorder and dependence are believed to be influenced by alcohol's pharmacological effects on the mesocorticolimbic system (Dehaan et al., 2013). As part of the mesocorticolimbic system the ventral tegmental area (VTA) is involved in the regulation of motivation and goal-directed behavior (Doyon et al., 2020). It is known that VTA neurons mainly synthesize dopamine, GABA, and glutamate (Nair-Roberts et al., 2008). A number of studies have examined these neurons in animals under anesthesia, finding that the administration of ethanol induces a moderate dose-dependent increase in the firing rate above baseline and burst activity of dopaminergic neurons (Foddai et al., 2004). With a number of these studies being done under anesthesia there is the possibility however, that anesthetic agents could disrupt normal neural activity and ultimately the effect of acute ethanol administration.

Our colleague at the University of Pennsylvania, Dr. William Doyon, aimed to better understand the motivational properties of ethanol by determining how ethanol modifies these neural circuits in freely moving animals. Evidence of discrete electrophysiological effects of ethanol on dopamine neurons compared with other VTA cell types, suggested a complex role of the VTA in alcohol-induced responses in freely moving animals (Doyon et al., 2020). The initial experiments were designed to assess the effect of a slow i.v. ethanol infusion on the firing activity of putative dopamine and GABA neurons, and unidentified neurons in vivo (Doyon et al., 2020). There was difficulty however, in knowing the time course of ethanol due to the lack of data on an ethanol concentration profile in the VTA. Separate microdialysis

experiments were conducted to overcome this limitation and to create a time course profile of ethanol in the VTA following an i.v. infusion. The analysis of dialysate and tissue concentrations were critical for final interpretations of how acute ethanol effects VTA neural activity.

Methodology

Male Long-Evans rats (300–450 g) were used for both in vivo recordings and microdialysis experiments. Multiple tetrode electrodes were used for in vivo recordings of the midbrain (VTA) and were conducted by Dr. Doyon. See Doyon et al. (2020) for electrophysiological effects of ethanol on the VTA.

In vivo microdialysis and gas chromatography were used to quantify ethanol concentrations directly in the midbrain during and after the ethanol infusion (1.5 g/kg administered over 9 min). This approach allowed us to determine the rising and falling phase of ethanol over time. For the microdialysis experiments each animal was implanted with a microdialysis guide cannula aimed at the VTA, and an intravenous catheter was also surgically placed into the jugular vein. Surgeries and sample collection were conducted at the University of Pennsylvania (Doyon et al. 2020).

Ethanol Analysis in Dialysates and Blood Samples

Both dialysis and blood samples were shipped overnight from Philadelphia, PA to Austin, TX. Immediately after collection of each dialysis sample, 4 µl was transferred to a 0.5 ml cryovial, and the vial was sealed (Sarstedt, Newton, NC, USA; part numbers 72.785 and 65.716). Blood samples (50 µl aliquots of whole blood) were diluted 1:10 in saturated NaCl

solution and were transferred into a 2 ml glass vial and sealed. Two separate ethanol standards (40 mM) were included to be used as controls. Samples were shipped in a Styrofoam box with frozen cold packs to minimize loss of ethanol from the vials due to the volatile nature of ethanol. Once received, all samples were inspected for damage and transferred to a lab refrigerator. Dialysis samples were centrifuged at room temperature for 10 min at 5,000-10,000 rpm. Then 2 µl of the sample were transferred to a 2 ml autosampler vial for analysis. Additionally, 50 ul of each blood sample were transferred into autosampler vials for further analysis. The 40 mM ethanol standards were used to quantitate the ethanol concentrations in the dialysis and blood samples.

Ethanol concentrations in dialysates and blood were determined using gas chromatography with flame ionization detection using methods modified from Carrillo et al. (2008). The gas chromatograph (Scion 436; Bruker, Netherlands) had a Bruker CP-8400 autosampler attached for injections. Samples were heated for at least 45 min before analysis. The stationary phase was an HP Innowax capillary column (30 m x 0.53 mm x 1.0 um film thickness). CompassCDS (v. 3.0.0.68, Bruker, Netherlands) software was used to record and analyze all chromatograms.

Results

After each experiment animals were perfused and the brain was analyzed for probe placement in the midbrain. During the infusion of ethanol in the microdialysis experiment, we expected to see a linear increase in dialysate ethanol concentrations. A linear regression of ethanol concentration with respect to time was conducted on dialysate samples taken during

the intravenous infusion of ethanol. Three out of four rats (n = 3) showed a significant correlation for linear regression ($r = 0.98\text{-}0.99$; $p < 0.001$). A fourth rat was excluded because the dialysate ethanol concentration did not follow a linear increase, which was reflected by a low *r*-value (0.02) that was not statistically significant ($p = 0.97$). It is possible that unknown damage occurred during the shipping process for the fourth rats' dialysate samples.

During dialysate collection two-minute sampling intervals were used. We found that ethanol levels increased during ethanol infusion and peaked at the 10 minute mark (figure 5).

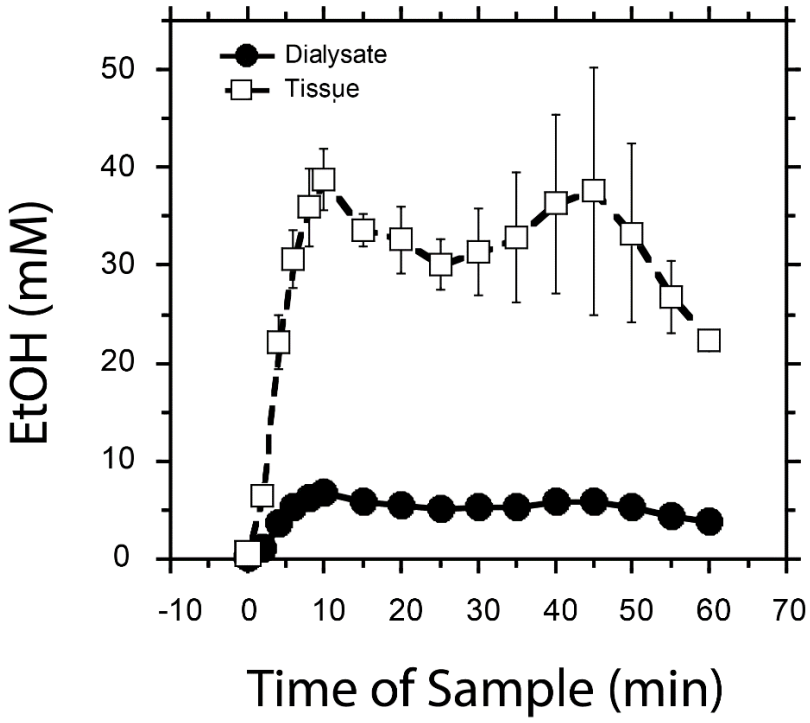

Figure 5: Time Course of Ethanol in Dialysate and Tissue. The graph shows the time course of ethanol over a sixty-minute time period in the VTA (n=3). Some error bars are not shown due to small SEMs (error bars represent the standard error of the mean).

To estimate the brain ethanol levels blood samples were collected after dialysis from each rat (n=3) at 70 minutes after the i.v. ethanol infusion began. The ethanol concentrations in the blood were used to determine the in vivo extraction fraction for ethanol, with the assumption that blood and brain ethanol levels are equivalent at this time point. The peak ethanol levels in the midbrain were estimated to be 39 ± 3 mM at 10 minutes and declined to 27 ± 3 mM at 60 minutes (figure 6)

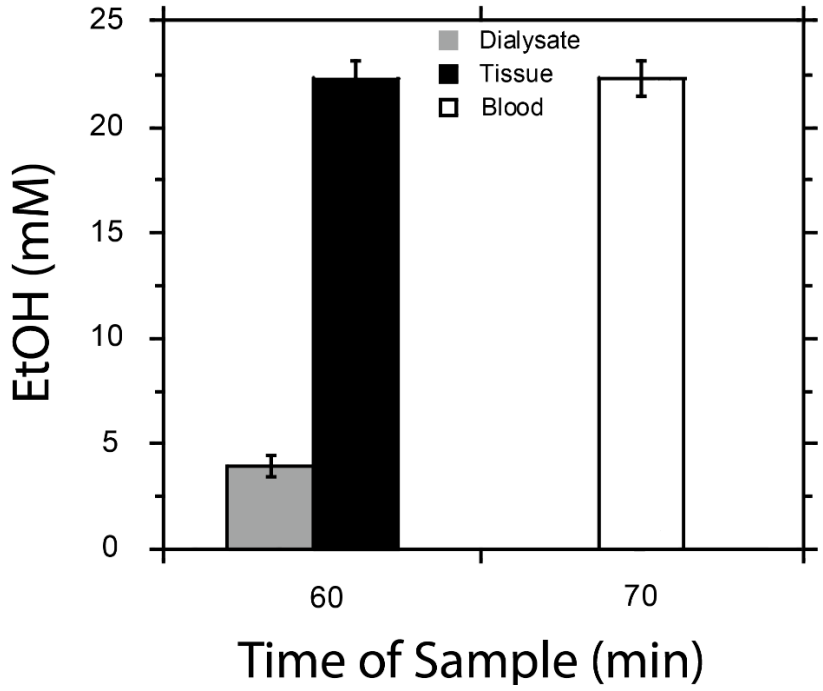

Figure 6: Dialysate, Tissue and Blood Ethanol Concentrations. The graph shows ethanol concentrations in dialysates and tissue at the end of the microdialysis collection (sixty minutes). As well, ethanol concentrations in blood samples are indicated after microdialysis collection at seventy minutes (n=3). (error bars represent the standard error of the mean).

Discussion

Published literature in the field has examined regional distribution of ethanol throughout the brain. Erickson (1976) used intracerebral perfusion cannulae to measure ethanol in several brain regions of freely moving rats. This study was the first to show that there is a significant differential distribution of ethanol in the rat brain after a 1 g/kg dose of ethanol, and that this unequal brain ethanol distribution may influence the behavioral effects of the drug. Although this study was able to distinguish differences in brain regions, he did not examine the VTA. This study made advancements in methodology on how we can measure ethanol in given brain region, specifically a time course was able to be determined in a given brain region in each animal. Tissue extraction experiments are also common throughout the field, however, are not the best method when creating a time course because multiple animals need to be used for each time point. This method is not only labor intensive but requires many animals. Microdialysis allows us the ability to collect serial samples from an animal and can be used to conduct a dynamic study on ethanol peak levels, as well as the decline of ethanol in brain regions over a given period. Past studies in our lab have successfully used this methodology to create a time course of ethanol in the striatum and the nucleus accumbens (Gonzales et al., 1998, Howard et al., 2008). By utilizing this method when looking at the VTA we are able to understand the relationship between the pharmacological and behavioral effects of ethanol reaching the brain during and after exposure. In addition, knowing the time course can provide additional insight of neurobiological mechanisms of ethanol that might occur during that time period in a brain region where that probe was placed.

Using this methodology we have now, for the first time, shown how ethanol concentrations change within the VTA during and after intravenous administration. The collection and analysis of dialysates were crucial for final interpretation of how acute ethanol affects VTA neural activity in our colleague's in vivo tetrode experiments. Independently, our analysis was novel to the field in its ability to create a time course of ethanol in the VTA, showing peak levels of ethanol to be at ten minutes and declining over the course of sixty minutes. This is an important advance for the field that could aid in future interpretations in experiments exploring ethanol effects in the midbrain. In general, our method in microdialysis collection and analysis could also be used to quantitate ethanol in other brain regions. One advantage of using microdialysis for ethanol collection is that the probes are versatile and can be aimed or modified for specific brain regions. Being able to quantify ethanol allows for a greater understanding of how long ethanol is present and what concentrations can potentially affect neuronal networks over time in a brain region.

Conclusion

As stated throughout the thesis individuals with AUD continue to suffer from a disruption of several neural systems underlying motivation, emotion, and cognition. AUD patients exhibit not only elevated motivation for alcohol but heightened stress and anxiety (Morrow and Creese 1986; Vazey et al., 2018). The high prevalence of AUD, alongside the wide range of effects of ethanol on biological, physiological, and neural functions continues to push the need for alcohol research. AUD research has aimed to not only better understand site-specific actions of ethanol on several brain regions, but to also create treatments that can alleviate the multitude of symptoms that come along with AUD. The goal of my thesis was to be able to add to the field by providing novel techniques and information that can aid alcohol dependence research.

In the first chapter the data collected from our ethanol vapor study showed that we were able to successfully induce alcohol dependence and withdrawal through a short ten-day vapor exposure. We were able to maintain all ethanol exposed Long Evans rats intoxicated throughout the ten days at a target BAC of at least 0.15 g/dl. In addition, all ethanol exposed rats showed signs of moderate withdrawal at four and eight hours after the chambers were turned off, with no signs of seizures. Through our data we demonstrate how important it is to consider individual differences in each animal that could potentially affect how severely intoxicated they become during each exposure. For example, severe intoxication could ultimately lead to weight loss during the experiment. This vapor study is currently the shortest vapor exposure model that has been able to successfully induce alcohol dependence over ten days in adult rats.

Lastly, in the final chapter I aided a colleague, Dr. William Doyon, by using gas chromatography to analyze and create a time course profile of ethanol in the VTA following

an i.v. ethanol infusion. Our analysis was novel to the field in its ability to create a time course of ethanol in the VTA, showing peak levels of ethanol to be at ten minutes and declining over the course of sixty minutes. In general, our method in microdialysis collection, analysis and use of an extraction fraction could also be used to quantitate ethanol in other brain regions. Being able to successfully quantify ethanol in other brain regions will allow for a greater understanding of how long ethanol is present and what concentrations can potentially affect neuronal networks.

Data collected from all these two chapters independently bring novel findings to the field that could strengthen techniques used in future alcohol dependence studies, as well as aiding in a better interpretation of the data. Our lab aims to continue to research alcohol dependance and withdrawal with the goal to examine possible neuromodulator targets that could be used as possible treatments for AUD in the future.

It is well known that AUD affects a significant proportion of the population, and the make up of the individuals spans all demographic categories, and therefore it is not surprising that there are individual differences in susceptibility and symptoms of AUD that are expressed. One of the goals of research is to gain an understanding of all the individual mechanistic functions of neurotransmitters, and how those influence the synaptic networks throughout the brain. The results of this research should provide advancements that are able to lead to manageable treatments for individuals suffering from addiction. Whether an individual is more susceptible to the reward, withdrawal or is suffering from an existing disorder, such as anxiety, treatment will ultimately end up working for one group and not for another. The results from this thesis have provided some new insight into factors that influence ethanol related behaviors and could be useful for future mechanistic studies of AUD.

Appendix: Possible Effects of Surgery on the Estrous Cycle

The following appendix aims to explore additional work done throughout my master's degree. It is important to state that additional studies would be needed to make a conclusion about the effects of surgery on the estrous cycle.

The short length of the estrous cycle of rats makes them ideal for investigating changes occurring during the reproductive cycle. The estrous cycle lasts four/five days and is characterized as: proestrus, estrus, metestrus and diestrus, which can be determined according to the cell types observed in the vaginal smear (Marcondes et al., 2001). Ovarian hormone fluctuations have been known to lead to several behavioral changes in female rats. For example, early studies have reported changes in activity in behavioral paradigms such as the novel open-field arena, as well as decreased locomotion, during different phases of the estrous cycle; (Gray and Levine, 1964). In addition, there is evidence that environmental stimuli can also affect estrous cycling (Gray and Levine, 1964). The importance of the estrous cycle has led to an increased interest in the possibility that the stages of the cycle can contribute to differences between males and females in various physiological measures. This interest has ultimately led to the more common practice of including female rats in an experiment. However, there is still concern of other possible variables such as stress from surgery that could affect the reproductive cycle of female animals and the consequent data collected.

Our lab aims to examine the effects of ethanol on neurochemical components of the brain in both male and female rodents. Despite the known behavioral effects of the estrous

cycle, it is not known if ovarian hormone fluctuations would affect neurochemical concentrations. The following study was conducted to examine the effects of a common cannulation surgery done in our lab and how it could potentially affect female rodents before conducting a microdialysis experiment.

Methodology

Surgery

Cannulation was carried out according to Duvauchelle et al. (1998) and Howard et al. (2008). Rats were initially anesthetized with 5% isoflurane in an induction chamber. Once the rat lost hind limb reflex, the isoflurane anesthetization was continued via a nose cone and lowered to 2.5% or a concentration low enough to keep the rat properly anesthetized. Rats were placed into a stereotaxic apparatus to implant the microdialysis cannula (21 gauge, 8 mm below the pedestal, Plastics One, Roanoke, VA). The skull was leveled, and a small hole was drilled above the brain region of interest (These females were involved in a pilot microdialysis experiment in our lab. Surgery was done by post-doctoral fellow Dr. Saul Jaime.). The guide cannula was secured onto the skull along with anchor screws with dental cement. An obturator was placed into the guide cannula to prevent entry of foreign matter into the cannula. The health status of each rat was monitored every day, and they were given a week for postsurgical recovery before any further experimentation or handling was conducted.

Lavage

The assessment of changes in vaginal epithelial cells was used to track the estrous cycle. All female Long Evans rats underwent daily vaginal lavage before and after cannulation

(except during the week of recovery from surgery). Female rats were covered and secured with a towel during the procedure to prevent any harm or excess stress. Daily samples were then taken with a 1 ul pipette between 10 am and 11 am. The pipette tip was inserted into the vaginal orifice and flushed with room temperature saline. The sample was then placed on a previously labeled designated slide for each individual female rat (slides: Fisherbrand Frosted Microscope Slides Precleaned, 12-550-343 (3" X 1" x 1.0mm)) for further cytological analysis. Once collection from all female rats was complete, each slide was examined with a microscope (Olympus Tokyo Ck) to characterize each vaginal smear. Goldman (2007) was consistently used to identify the stage of estrous cycle of each slide. Each vaginal smear was then left to dry overnight to be stored the next day in a microscope box for future hydration if necessary.

Statistics

Pre-surgical and post-surgical weights were analyzed using repeated measures of analysis of variance (ANOVA). Post hoc analyses (using Bonferroni corrections) were done to follow up on any significant findings from the ANOVA. For this experiment, significance was designated when $p < 0.05$ for main effects, interactions, and post hoc analyses (with Bonferroni corrections).

Results

Estrous Cycling

Lavages were performed on eight female Long Evans rats for five days before undergoing cannula surgery. Of those eight, five female rats were identified as cycling prior to surgery and included in the study (see Table 3).

	SJF10	SJF13	SJF15	SJF16	SJF17
2/23	D	P (e)	E	D (p)	D (p)
2/24	E	E	E	M	D
2/25	E (m)	E	E	E	M
2/26	M (d)	M (d)	E	E	E
2/27	P	D	P	D	D
2/28	E	E	E (m)	D	M
CYCLING	Yes	Yes	Yes	Yes	Yes
	Surgery and Recovery				
3/6	Bad Sample	E	M	M	P (e)
3/7	M	P	E	E	D
3/8	E	M	D	E	D
3/9	D	E(M)	D	M	E
3/10	D	E	D	D	E
3/11	P	E	D	E (m)	D
3/12	E	D	M	P	P
3/13	M	D	M	E	M
3/14	Microdialysis (No Sample)	Microdialysis (No Sample)	D	P	E
CYCLING	Yes	Yes	No	Yes	Yes

Table 3: Estrous Cycle Before and After Cannula Surgery

Table 3 shows that all five female rats cycled through proestrus at least once before surgery. After cannula surgery and a week of recovery, we found that four of the female Long Evans rats continued to cycle normally, while the fifth (SJF15) stopped cycling and remained in the metestrus/diestrus stage.

Weight Loss

Weights for each female rat were collected before and after cannula surgery. Notably, all five female Long Evans rats began to lose weight after surgery. Figure 7 shows the average daily weights pre-surgery and post-surgery. A repeated one-way ANOVA was conducted on all daily averages and showed that there was a difference in weights throughout the experiment. ($F(12,48) = 3.264$, $p < 0.05$). However, further post hoc analyses did not result in a significant difference in pre-surgery weights when compared to each post-surgery weight. Even though we did find an overall significant difference in weights, more in-depth post hoc analyses would be necessary to find the source. It is possible that the female rats experienced weight loss due to surgery but had already begun to recover the weight back during their recovery period. Therefore any effect on cycling would not be due to weight loss caused by surgery.

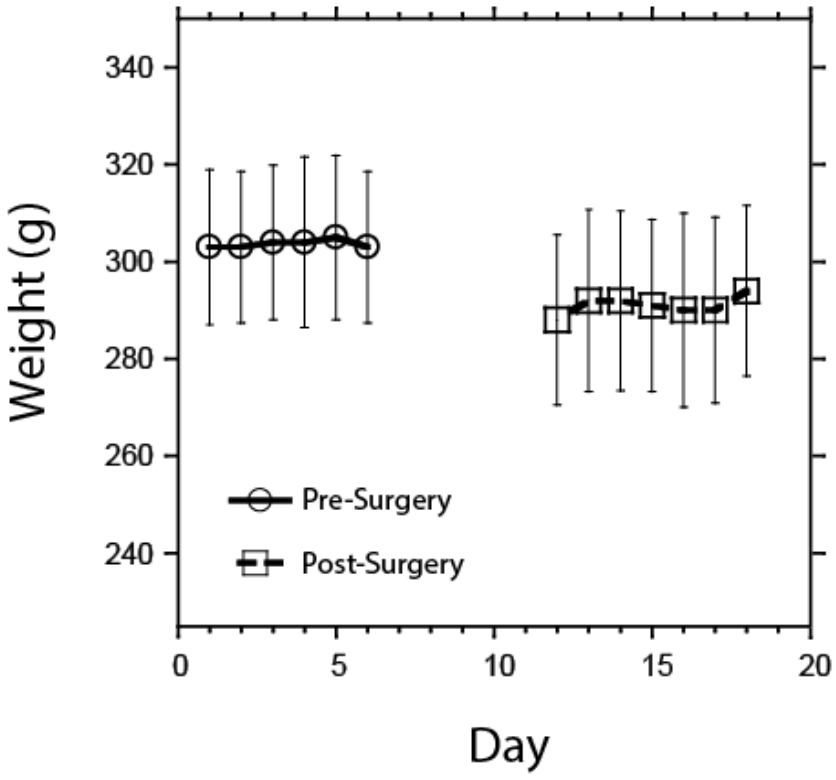

Figure 7: Female Weights Pre and Post Surgery. The following graph shows female (n=5) weights in grams pre-surgery and post-surgery. A difference in weights throughout the experiment was found.

Discussion

It is important to state that more data needs to be collected to make a definite conclusion on the effects of surgery on the estrous cycle. The data from the present pilot shows that all five female Long Evans rats were cycling prior to cannula surgery. After surgery and a week of recovery, we found that four animals continued to cycle while one remained in the diestrus/metestrus stage. Additionally, there was a significant difference in pre- and post-surgery weights. However, we were not able to identify the cause of this difference with the

post hoc tests used in this study to compare individual pre- and post-surgery weights. Our data points to the possibility that the cannula surgery was severe enough to cause weight loss in the beginning of the recovery period, but that the rats began to gain it back towards the end of recovery. As such, it is less likely that the initial post-surgery weight loss was the cause for only one rat to stop cycling.

Stress caused by cannula surgery is more than likely the cause that halted estrous cycling in that individual rat. Silva (2020) used guide cannulae for microinjections in female *Rattus norvegicus* and found that only seven out of thirty females continued to cycle one day after surgery. Additionally, twenty-one rats needed up to sixteen days to cycle again, while the remaining two rats were unable to cycle and therefore taken out of the study. Robinson (2002) was also able to successfully examine the effects of gender and estrous cycle on the pharmacokinetics of ethanol through microdialysis by allowing females rats an appropriate time to recover from the surgery. Our data is consistent with the previous findings showing that invasive surgical procedures can cause transient effects in female rats. More work is necessary to explore the possible effects of surgery on the estrous cycle, however it is suggested that future studies should consider monitoring rodents before an experimental treatment for at least two cycles to better confirm that rats are cycling. In addition, female rats should be given proper recovery time, from one to two weeks, which would not only allow any weight loss to be gained back but would give them enough time to go through a few cycling periods.

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